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**Short Synopsis**
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**TITLE: Enhancement of Secondary Metabolite Production in Callus Cultures of *Glycyrrhiza glabra* Linn. through Elicitation**

**DEPARTMENT OF BIOTECHNOLOGY**

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**ABSTRACT**

Plants produce a number of phytochemicals in response to environmental stress. These, known as secondary metabolites are widely used as commercial and pharmaceutical products. The chemical synthesis of most of the phytochemicals is not feasible due to the complex structure and chirality exhibited by these compounds. In vivo production of secondary metabolites is often not consistent due to several environmental factors, however, plant cell cultures offer a good alternative for consistent production of desired secondary metabolites.

*Glycyrrhiza glabra* Linn. (Licorice) is a medicinal plant of family Fabaceae which possess a wide range of phytochemicals and is well known for its pharmaceutical properties.

This research will be focused on the enhancement of secondary metabolite production in *Glycyrrhiza glabra* callus cultures using various elicitors. Callus cultures of *Glycyrrhiza glabra* will be established and maintained using nutrient media supplemented with growth hormones in various concentrations and combinations. Elicitation of cultures will be done using various biotic and abiotic elicitors and subsequently elicited cultures will be evaluated for enhanced production of secondary metabolites.

**Key words**: *Glycyrrhiza glabra*, callus culture, cell suspension culture, secondary metabolites, elicitation

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## 1. INTRODUCTION

Plants synthesize and store a wide variety of biochemical compounds called secondary metabolites which are conventionally recognized as pharmaceuticals, flavors, fragrances, dyes, pigments, pesticides, food additives and many more. Most secondary metabolites are metabolically induced in plants in response to environmental stresses and hence play defensive role enabling protection to plant from various biotic and abiotic factors. Secondary metabolites can be broadly classified as terpenoids, alkaloids and phenolic compounds which are synthesized through their specific metabolite pathways and possess specific structural and functional characteristics.

Among these, Terpenes are the most widespread and chemically diverse group of natural phytochemicals whose structures are derived from isoprene units. These are synthesized through mevalonate and non-mevalonate or 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (DOX-MEP) pathways where in isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) combine to yield geranyl pyrophosphate (GPP), leading to the formation of monoterpenes. Sesquiterpenes and triterpenes consist of three isoprene units and six isoprene units respectively and are derived from farnesyl pyrophosphate (FPP). Terpenes are extensively applied in industrial sectors as flavours, fragrances and in cosmetics and food additives manufacturing. Many terpenes are also used for medicinal purposes as they have biological activities against cancer, malaria, inflammation and variety of infectious diseases (Roslin *et al*. 2011).

Phenolic compounds are a large class of plant secondary metabolites derived from phenyl propanoid pathway. These compounds have diverse structures and contribute to the colour of flowers, fruits and vegetables. Various bioactivities of phenolic compounds are responsible for their chemopreventive properties such as anti-oxidant, anti-carcinogenic and anti-inflammatory effects (Huang *et al*. 2010). Another class of secondary metabolites is alkaloids. They often have pharmacological effects and are used as medicines or as recreational drugs. These are organic compounds that contain a nitrogen based heterocylic ring derived from aromatic amino acids and are synthesized from the shikimic acid pathway. Common metabolic precursors for both phenolic compounds and alkaloids are aromatic amino acids phenyl alanine and tryptophan.

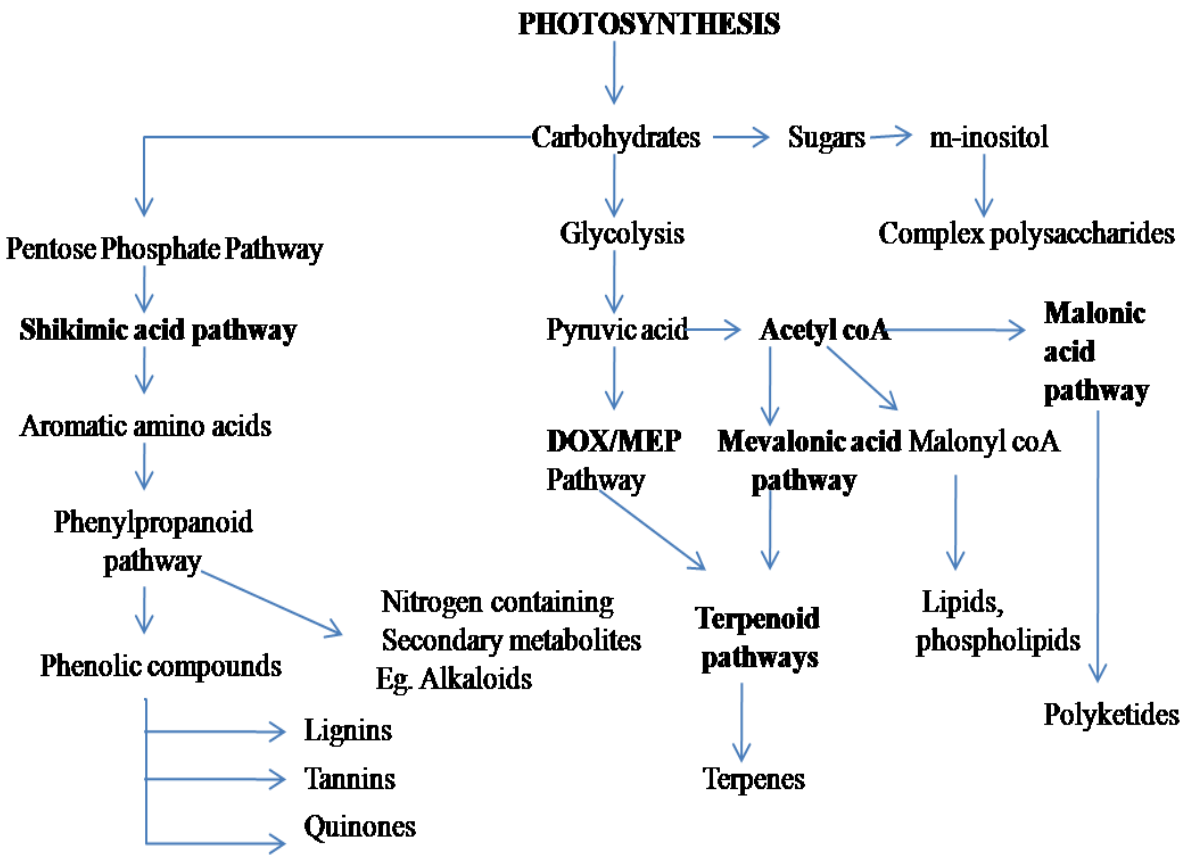



**Fig: 1 Schematic representation of basic biosynthetic pathways leading to the synthesis of secondary metabolites**

Biochemical synthesis of secondary metabolites for industrial use is often not feasible due to complex metabolic pathways, complicated structures and chirality exhibited by these compounds. The commercial demand of these compounds can only be met by obtaining them directly from field grown plants. However, most plants accumulate secondary metabolites in small amounts in specialized tissues probably after attaining a certain stage in their life cycle. Apart from this, the yields of secondary metabolites extracted from field grown plants are influenced by many factors like climate, pests and diseases which are difficult to control and in turn affect their consistent production, due to which the commercial exploitation becomes a challenging task. Efficient extraction of desired compounds may require complete harvesting of the plant parts or whole plant. Blind harvesting of medicinal plants has led to extinction of several valuable plant species. Based on the International Union for Conservation of Nature and Natural Resources' (IUCN's) Red List Categories, the Indian government assessed the status of 359 wild medicinal plants and 93 percent of plants were found to be either threatened,

vulnerable, endangered or critically endangered, primarily due to their overexploitation Biotechnological approaches, specifically plant tissue cultures, are found to be good alternatives to overcome these demerits and offer consistent yield of secondary metabolites for commercial use. Plant cell and tissue cultures are capable of producing specific phytochemicals at a rate similar or superior to that of intact plants (Aijaz *et al.* 2011). Moreover, the biosynthetic capacity of cultured plant tissue can be enhanced by regulating environmental factors, as well as by artificial selection or induction of variant clones for high productivity. Several phytochemicals localized in morphologically specialized tissues or organs of native plants have been produced in culture systems not only by inducing specific organized cultures, but also by undifferentiated cell cultures (Aijaz *et al.* 2011).

In the process of plant tissue culture, explants are cultured under appropriate physiological conditions and the desired product is extracted from the cultured cells/tissue. Recent developments in plant tissue culture techniques and their processing have shown promising results to improve the productivity to many folds and has made it possible to gradually replace the whole plant cultivation as a source of useful secondary metabolites **(**Chattopadhyay et al., 2002). Today, various tissue culture techniques are being used to enhance yield of secondary metabolites by invigorating plant defence and triggering stress response in plant cells with the help of elicitors. Elicitors are being used as an enhancement strategy in plant secondary-metabolite synthesis as they play an important role in stimulating the biosynthetic pathways leading to enhanced production of commercially important compounds. This provides an opportunity for intensive research in the field of plant sciences not only for exploitation of plant cells for increased yield of secondary metabolites, but also for investigation of plant defence mechanism and regulation of secondary metabolism.

The medicinal plant *Glycyrrhiza glabra* has possess wide range of important phytoconstituents and provide a good scope for research on the production and enhancement of its secondary metabolites. *Glycyrrhiza glabra* commonly known *as* licorice is a hard herb or under shrub of family Fabaceae. It is an economically useful medicinal plant native to Mediterranean region and parts of Asia and is a source of large number of secondary metabolites of therapeutic value. Many ayurvedic preparations containing licorice such as *yashtyadi churna*, *yashtimadhvadya taila*, *brihat ashwagandha gritha*, *pippalyadi taila* and *vridhihara lepa*, have age long therapeutic uses in cough, respiratory disorders, hairfall, baldness, piles, gout, weakness

in lower back and lower limbs, lumbar and cervical spondylosis, constipation and allergic rhinitis (Bhakti *et al*. 2009; Meena *et al*. 2010). *Glycyrrhiza glabra* extracts have been investigated for their therapeutic benefits against many harmful viruses including *Herpes simplex* virus (HSV), Human papilloma virus (HPV) and Human immunodeficiency virus (HIV) which have long latent period and are still incurable. The extracts of licorice are also reported to have potential benefits against the Hepatitis B virus and seem to be promising to treat chronic Hepatitis (Rathee *et al*. 2010). The clinically proven activities of licorice such as anti ulcer, anti microbial, anti asthmatic, anti diuretic and anti hepatotoxic activity (Vispute and Khopade, 2011) are attributed to the wide range of phytochemicals possessed by licorice.

Glycyrrhizin, one of the active constituent of this plant is a prescription drug used in the treatment of liver and allergic diseases. It is manufactured in the form of injection (Stronger Neo-Minophagen®C) and tablet (Glycyron®) which are available in India and many other countries (Hayashi and Sudo, 2009). Glycyrrhetinic acid is also an active constituent of the prescription drug used in the treatment of peptic ulcers. It has also been used as a cure to atopic dermatitis, pruritis and cysts due to parasitic infestations of skin (Saeedi *et al*. 2003). In modern medicine, licorice extracts are often used as flavouring agents to mask bitter taste in tonics and as an expectorant in cough syrups (Kanimozhi and Karthikeyan, 2011). Gels containing glycyrrhizin are used for the treatment of oral diseases and genital lesions caused due to *Herpes simplex* virus (Segal *et al*., 1987, Varsha *et al*., 2009). Bioactive constituents of *Glycyrrhiza glabra* are being explored for their potential used as anti cancer and anti HIV drugs. This plant is certainly promising candidate for providing new derivatives of pharmaceutically active constituents which can be evaluated for pharmacological use as future drugs for prevention and cure of large number of ailments.

## 2. LITERATURE REVIEW

Since last few decades, numerous strategies have been adopted for the production of pharmaceutically important secondary metabolites from medicinal plants using *in vitro* system like plant tissue cultures. *Glycyrrhiza glabra* is one such medicinal plant of pharmaceutical importance which has been used in medicines for more than 4000 years.

Cell and tissue cultures of *Glycyrrhiza glabra* act as good source of a wide range of phytochemicals and therefore can be seen as efficient systems for the *in vitro* production of valuable secondary metabolites. In many studies *in vitro* cultures of *Glycyrrhiza glabra* including callus cultures and cell suspension cultures have been established and evaluated for their secondary metabolite production. Hyashi *et al*. (1990) investigated production of triterpenoids like butulinic acid, β-amyrin and lupeol in callus and suspension cultures of *Glycyrrhiza glabra*. In 1993, Arias-Castro *et al*. established the cell-suspension cultures of *Glycyrrhiza glabra* and studied the growth characteristics. Tailang *et al.* (1997) carried out mutation induced bioproduction of glycyrrhetinic acid from *Glycyrrhiza glabra* callus cultures.

Now it is well known that *Glycyrrhiza glabra* contains a number of important phytochemical constituents such as triterpene saponins, flavonoids, isoflavones, pectins, polysaccharides, amino acids, mineral salts, simple sugars (Obolentseva *et al*. 1999) and coumarins like liqcoumarin, glabrocoumarone A and B, herniarin, umbelliferone, glycocoumarin, licofuranocoumarin, licopyranocoumarin, glabrocoumarin (Nomura *et al.*, 2002). The application of in vitro system for commercial production of such pharmaceutically valuable secondary metabolites can be justified only if it turns to be highly productive and at the same time cost effective. In order to obtain persistent production and high yields of secondary metabolites from cultured cells, elicitation is one of the most successful methods used for the induction of these products. Some elicitation studies have been done on the tissue cultures of *Glycyrrhiza glabra* and other species of *Glycyrrhiza* for enhancement of secondary metabolites. Hayashi *et al*. (2003) studied the elicitation of soyasaponin production by methyl jasmonate in cultured cells of *Glycyrrhiza glabra* through upregulation of enzymes involved in its biosynthesis. They found that methyl jasmonate upregulated few enzymes involved in soyasaponin biosynthesis. Yeast extract was also found effective in promoting the betulinic acid and soyasaponin accumulation in cell cultures of *G.glabra* (Hayashi *et al*. 2005).

Glycyrrhizin, a triterpenoid saponin is a major bioactive component of *G.glabra* which is 50 times sweeter than sugar and possesses a wide range of pharmacological properties. Few more triterpenes of pharmaceutical importance present in *G.glabra* are 18β-glycyrrhetinic acid, glycyrretol, liquiritic acid, glabrolide, isoglaborlide and liquorice acid (Isbrucker &

Burdock 2006). Mousa *et al*. (2007) reported the production of Glycyrrhizin in cell-suspension cultures of *Glycyrrhiza glabra.* Scale up of hairy root cultures of *Glycyrrhiza glabra* to bioreactor levels was reported by Mehrotra *et al*. in 2008. Studies on glycyrrhizin production using callus cultures of *Glycyrrhiza glabra* has been done by Wongwicha *et al*.2008. Few researchers have used exogenously supplied elicitors to stimulate production of secondary metabolites in tissue cultures of *Glycyrrhiza glabra*. Shabani *et al*. (2009) reported that 0.1-2 mM methyl jasmonate and 0.1 and 1 mM salicylic acid enhanced the production of glycyrrhizin by 3.8 and 4.1 times respectively, in the roots of in-vitro grown *G.glabra* plants. Zhang & Ye 2009 isolated more than 300 flavonoids from *Glycyrrhiza* species, of which flavanones and chalcones are the main types. Enhancement of flavonoid in hairy root cultures of *Glycyrrhiza* was achieved through a combined approach of elicitation and genetic engineering by Zhang *et al*. (2009).

Paraseimehr *et al*. (2009) and Patel *et* al. (2011) were successful in their attempts to establish cell-suspension cultures of this plant. Attempts of enhancement of Glycyrrhizin in *Glycyrrhiza inflata* hairy root cultures have been done by Wongwicha *et al*. (2011). Shirazi et al. (2012) have investigated the production of isoliquirtigenin in hairy root cultures of *Glycyrrhiza glabra.* Recently, some reports on the in vitro plant regeneration and conservation of this plant were found to be documented (Kuldeep Yadav and Narender Singh, 2012; Verma *et al*. 2012; Srivastava *et al*. 2013). However, literature survey revealed that studies on secondary metabolite production and elicitation in callus cultures of *Glycyrrhiza glabra* are meager.

## 3. DETAILED DESCRIPTION OF BROAD AREA

*Glycyrrhiza glabra* is included in the planning commission list (March, 2000) of most used plants in Indian system of medicines and is at the verge of being endangered due to over exploitation. Most of the pharmaceutically important secondary metabolites of *Glycyrrhiza glabra* are synthesized in their roots and are accumulated in their roots in considerable quantity only after attaining certain years of maturity. In vivo extraction of these metabolites from roots of plant is difficult and requires harvesting of matured roots often involving complete uprooting of plant after which there are minimal chances of its revival even if it is replanted, leading to

complete loss of this plant. In this regard, plant tissue culture can be seen as an alternative towards conservation of this plant without posing threat to biodiversity.

Plant cell and tissue cultures offer great opportunity for controlled production of a number of useful secondary metabolites. Plant cells are biosynthetically totipotent, which means that each cell in culture retains complete genetic information and hence is able to produce the range of phytochemicals found in the parent plant. Some of the distinct advantages of in vitro secondary metabolite production over in vivo production are enlisted as-

- Production of metabolites in tissue cultures is quite simple and can be manipulated to some extent.
- Tissue cultures offer a defined production system, which ensures the continuous supply of products with uniform quality and consistent yield.
- Interfering compounds that usually occur *in vivo*, can be avoided in tissue cultures.
- Higher yields of desired product can be obtained in shorter time.
- Extraction of the phytochemicals from tissue cultures is rapid and more efficient as compared to that of complex whole plants.
- Tissue culture is an efficient model to engineer the metabolic pathways and induce the production of desired secondary metabolites.

The need for establishment of tissue cultures of *Glycyrrhiza glabra* was realized due to higher demand of the plant material for production of its commercially valuable secondary metabolites used in pharmaceutical, cosmetic and confectionary industry. In the process of in vitro production of secondary metabolites, callus culture is a primary step for subsequent plant cell culture. Moreover, callus as an unorganized mass of undifferentiated cells exhibit great biosynthetic potential and is a good candidate for achieving higher degree of target metabolite production supported by media manipulation and elicitation. Rapid growth, easy maintenance and controlled metabolic behavior in vitro are the factors which make it indispensable to explore not only for enhanced production of secondary metabolites but also for biotransformation. The desired compounds can be isolated by extraction of cultured callus tissue using suitable solvent.

The amount of secondary metabolites produced in tissue cultures can be further increased to significant levels by adapting some enhancement strategies. Therefore, effect of different biotic and abiotic elicitors, culture conditions, precursor feeding and media manipulation for enhancement of secondary metabolite production in *Glycyrrhiza glabra* tissue cultures will be investigated and different process of extraction for obtaining maximum amount of these metabolites will be explored. Treatment of cell/tissue cultures with biotic and abiotic elicitors either alone or in combination has been a useful strategy to enhance phytochemical production in cell cultures (Karuppusamy, 2009). An 'elicitor' is a substance which when introduced in small concentrations to a living cell system, induces the biosynthesis of specific compounds. Elicitors can be produced endogenously in plant cells on encountering a physical, chemical or biological stress or can be supplied exogenously. Abiotic elicitors are of non-biological origin including inorganic salts containing ions such as $Cu^{2+}$, $Cd^{2}$ , $Ca^{2+}$etc and physical factors such as UV radiation, high or low pH and temperature etc. 'Biotic elicitors' are substances of biological origin such as polysaccharides derived from plant cell walls like pectin or cellulose and those derived from micro-organisms such as chitin, glucans, glycoproteins etc, whose functions are coupled to receptors and act by activating or inactivating a number of enzymes or ion channels (Namdeo at al., 2007) .

Induction of secondary metabolite biosynthesis in plant tissue cultures is often done by providing chemical or physical elicitors exogenously. Elicitors signal the biosynthetic pathway either directly or indirectly to initiate or enhance production of secondary metabolites. When the plant tissue comes in contact with an elicitor, the normal metabolism of the plant tissue is altered and synthesis of signal transducers and enzymes is induced that catalyze reactions in the defense-related pathways leading to production of phytochemicals (Stephanie Moss, 2006). The effect of elicitors depends on various parameters like concentration of elicitor, stage of culture growth at the time of elicitation, period of contact between elicitor and plant tissue, and the time course of elicitation.The enhanced production of the secondary metbolites from plant cell/tissue cultures through elicitation is thus in itself a potential area of research which could have important economic benefits for pharmaceutical industry. Tissue cultures in combination with elicitation can be very good models for scale up of secondary metabolite production in *Glycyrrhiza glabra* and hold great promise for a viable industrial application.

## 4. OBJECTIVES

The focus of present study is the enhancement of secondary metabolite production in *Glycyrrhiza glabra* cultures using various elicitors, with the following objectives:

- To Characterize various secondary metabolites present in different parts of *Glycyrrhiza glabra.*
- To Optimize the extraction procedures and conditions for obtaining higher yields of secondary metabolites from *Glycyrrhiza glabra* roots in vivo.
- To establish and maintain the callus cultures of *Glycyrrhiza glabra*.
- To Extract and characterize the secondary metabolites present in *Glycyrrhiza glabra* callus cultures.
- To carry out elicitation of tissue cultures of *Glycyrrhiza glabra* for enhanced production of target secondary metabolites.
- To optimize the process of enhancement of target metabolites in *Glycyrrhiza glabra* callus cultures

## 5. METHODOLOGY

### 5.1. Preparation of crude extract of the roots, stem and leaves of in-vivo grown plant and their partial purification

Crude extracts of dried leaves, stem and roots of Glycyrrhiza glabra will be prepared using a general method of extraction of different secondary metabolites described by Harborne (1999), Handa *et al*., (2013) , and Marghitas *et al.,* (2007).

### 5.2. Qualitative and Quantitative analysis of Secondary metabolites in roots of *Glycyrrhiza glabra* .

The secondary metabolite content in the plant extracts will be analyzed quantitatively and quantified by using the Chromatographic and spectrometric methods like High performance liquid chromatography (HPLC) (Esmaeili et al., 2006) and Gas Chromatography-Mass Spectrometry (GC-MS) (Montoro *et al.,* 2006 and Farag *et al.,* 2012)

### 5.3. Establishment of Callus Cultures

Callus cultures will be established using different expalnts in Murashige and Skoog (MS) medium supplemented with different combinations of plant nutrients and growth hormones based on the method used by Wongwicha *et al*., 2008

### 5.5. Biotic and Abiotic Elicitation in Callus cultures

The callus cultures will be elicited with different biotic and biotic elicitors prepared at different concentrations as described in other reports (Hayashi et al., 2005; Karwasara *et al*., 2010; Ghorpade *et al.,* 2011) and their effect on secondary metabolite production and plant cell growth will be studied.

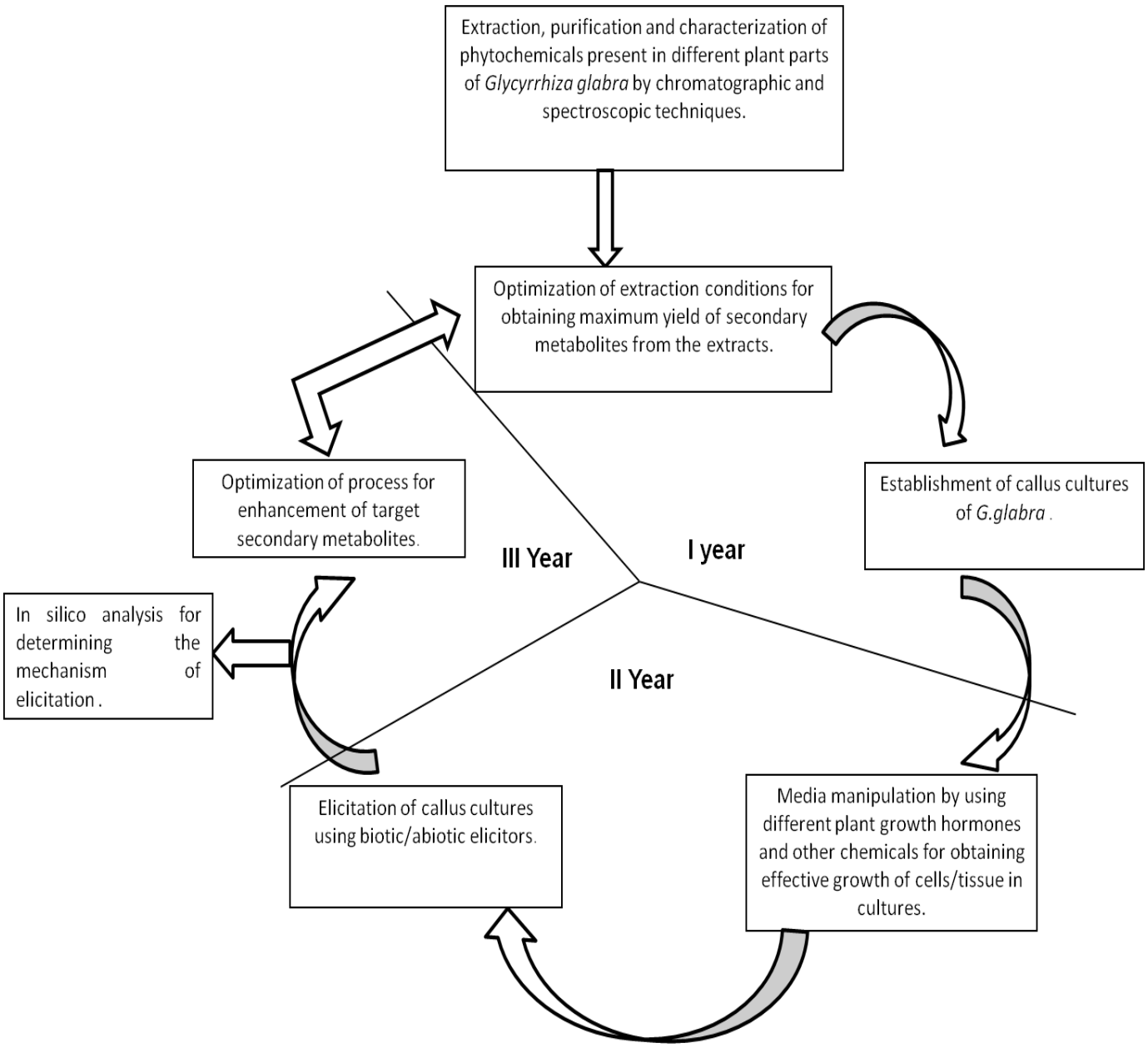
**5.7. Extraction, purification and characterization of secondary metabolites from callus cultures.**

Secondary metabolites will be extracted purified from the dried cultures by adopting the procedures described by J.B. Harborne (1999) and will be quantitatively analyzed chromatographic and spectroscopic methods.

**5.8. Optimization and standardization**

Based on the above methodologies, the conditions for obtaining maximum yield of secondary metabolites from callus cultures will be optimized after thorough statistical analysis and a standardized protocol will be established.

## 6. RESEARCH PLAN


Extraction, purification and characterization of phytochemicals present in different plant parts of *Glycyrrhiza glabra* by chromatographic and spectroscopic techniques.
Optimization of extraction conditions for obtaining maximum yield of secondary metabolites from the extracts.
Establishment of callus cultures of *G.glabra* .
I year
Media manipulation by using different plant growth hormones and other chemicals for obtaining effective growth of cells/tissue in cultures.
II Year
Elicitation of callus cultures using biotic/abiotic elicitors.
III Year
In silico analysis for determining the mechanism of elicitation .
Optimization of process for enhancement of target secondary metabolites.

## 7. EXPECTED OUTCOME

The expected outcome of this work is enhancement in production of target metabolites from callus cultures of *Glycyrrhiza glabra* as compared to in vivo production.

Different cultures have different physiological requirements of growth and metabolism. To succeed in establishing the callus cultures of *Glycyrrhiza glabra*, several essential conditions like nutrient composition, temperature, light and concentration of plant growth regulators will be taken in to consideration and will be optimized. For plant cell culture to be economically feasible, certain methods have to be adopted that would allow for consistent generation of high yields of secondary metabolites from cultured cells. This will involve the use of biotic and abiotic elicitors for induction of plant defense system and modification of plant metabolism in order to enhance the productivity of specific metabolites in plant cell cultures.

Since mechanism of action of each elicitor is considered to be complex and different from one another, it is difficult to predict the effect of elicitation on the plant cell culture due to their complex biosynthetic pathways. Therefore, several elicitors and their combinations will be experimented. The factors affecting elicitation process such as the concentration of elicitor used for the treatment, culture growth at the time of elicitation and time duration of elicitor treatment will be optimized and the changes in the growth and secondary metabolite production in tissue cultures in response to elicitation will also be evaluated.

The extraction of secondary metabolites from plant tissue cultures is also a crucial process as it requires the use of appropriate solvents and extraction conditions which have a greater impact on the yield of secondary metabolite.

## 8. SIGNIFICANCE OF WORK

*Glycyrrhiza glabra* is an important source of numerous compounds with different chemical structures as well as pharmacological properties and is in great demand throughout the world as medicinal and nutritional supplement. Continuous and irreplacable harvesting of this medicinal plant from its native flora has put it on the verge of being endangered. Application of tissue culture techniques for the production of secondary metabolites from this plant can not only be efficient in production of pharmaceutically valuable secondary metabolites but also lead to

conservation of the plant in its natural environment. Plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications, and in many cases, rigorously controlled plant *in vitro* cultures can generate the same valuable natural products and that too in large quantities. *Glycyrrhiza glabra* is one such commercially valuable plant and secondary metabolites from Glycyrrhiza roots have been commercially used in pharmaceutical, cosmetics and confectionary industries. Presence of many valuable chemical compounds in *Glycyrrhiza glabra* indicates that further research on enhancement in the production of these compounds through biotechnological methods like elicitation in tissue cultures could be of greater significance in future.

It is not only commercial significance that drives the research initiatives. The deliberate production of defined phytochemicals within carefully regulated *in vitro* cultures provides an excellent opportunity for in-depth investigation of biochemical and metabolic pathways, under highly controlled environmental conditions. Secondary compounds of *Glycyrrhiza glabra* have many pharmacological properties and are reported to promote the activation of interferon and inhibit the growth of several DNA and RNA viruses. Elicitation studies on such bioactive constituents of this plant will thus be a step towards the development of novel drugs efficient in treatment of various ailments which do not have a complete cure such as Cancer, HPV, HIV and HSV and can be used in cancer therapy and as antiviral drugs in near future.

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